6 Urinary measurement of 8-OxodG (8-Oxo-2′-deoxyguanosine)

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INTRODUCTION

The most abundant oxidative modification known in DNA is 8-hydroxylation of the guanine moiety. This modification is also mutagenic, preferentially resulting in G-T transversion mutation, demonstrated, for example, in the codon 248 of tumour supressor gene *P53*. Due to these observations, urinary excretion of the repair product 8-oxodG has attracted interest as a potential biomarker for the development of certain cancers (Loft and Poulsen, 1996).

Potential problems

- It has yet to be verified that urinary excretion of 8-oxodG is prognostic for development of cancer.
- Defined period of urine collection (24 hour or similar) is necessary for estimation of the excretion rate.
- Correction of spot urine samples by creatinine concentration does not correlate well with 24–hour urine excretion. Under certain circumstances (short trials, repeated measurements in the same individual, unchanged diet and muscle mass) short urine collection periods or even spot urine may be applicable. It is evident that group comparison (e.g. healthy versus diseased persons) cannot be done by spot urine and creatinine

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comparison, because of the large difference in creatinine excretion in the groups.

- Blank urine, i.e. urine without 8-oxodG, is not available and estimation of the urinary concentration must rely on sample addition or labelled internal standard.
- At present, ELISA methods do not appear to correlate with other methods. Values are high presumably due to insufficient specificity of the antibodies available.
- Synonyms for 8oxodG are: 7,8-dihydro-8-oxo-2′- deoxyguanosine, 8-oxo-2′-deoxyguanosine, 8-hydroxydeoxyguanosine, 8-hydroxy-2′-deoxy- guanosine, 8OHdG, oxo^{8}dG.
- It is not known if individual risks can be assessed by measurement of urinary 8-oxodG, or whether the potential as biomarker is limited to group comparison.
- The methodology for estimation of urinary 8xodG can also be used to estimate levels in tissue DNA. Particular care should be taken to avoid artificial oxidation in this case. For urine this problem is not of the same magnitude, due to low levels of non-oxidized bases and nucleotides.

METHODS OF ANALYSIS

The published values of urinary excretion of 8oxodG range from 110 to 600 pmol/kg body weight/24 h, and urinary concentrations range from about 1 nM to about 100 nM. There is no accreditation procedure or quality assurance established and the method is purely for research purpose.
The published methodologies include:

- enzyme-linked immuno-sorbent assay (ELISA)
- gas chromatography with selective ion monitoring (GCMS–SIM)
- high performance liquid chromatography with electrochemical detection (HPLC–EC)
- liquid chromatography with tandem mass spectroscopy (LCMS–MS)

The choice between the different methodologies depends mainly on the apparatus and know-how in the laboratory to set up the analysis, and the funds available.

The ELISA method is attractive because of the low price and simple technological requirements and because it relies on any brand of ELISA plate reader. The assays available so far overestimate levels by 3–5 times and at present the method cannot be recommended (Prieme *et al.* 1996). It is not further described in this chapter.

GCMS has been used in very few laboratories and is mainly used for estimation of 8-oxoGua in tissue DNA (see Chapter 4). HPLC–EC is the preferred methodology and is therefore described in detail.

LCMS–MS is a promising methodology, with the potential advantages of rapid analysis, simultaneous determination of other oxidative DNA base/nucleotide modifications and a sensitivity matching that of HPLC–EC. It has the advantage over GCMS of eliminating the derivatization step, thereby reducing the potential for introducing artificial oxidation.

HPLC–EC ANALYSIS

Apparatus and system set-up

- Two high-pressure HPLC pumps (Merck-Hitachi L-6000 and L- 6200)
- Column oven (40°C)
- Pulse dampener (LP21, Science Systems Inc.)
- Six-port fast switching valve (Valco)
- Electrochemical detector (ESA Coulochem II with 5011 high sensitivity cell)
- UV detector (Waters 440 UV detector, 254 nm)
- Autoinjector (Merck-Hitachi 655A-40)
- Extraction column (Spherisorb ODS2, 15 cm, 5 μm, Waters Denmark)
- Cation exchange (CE) column, 2 cm (Hamilton, Reno, Nevada)
- Analytical column (Nucleosil C18, 3 μm, Knaur, Germany)
- PC chromatographic data handling and integration software system (Merck-Hitachi D-6000 or higher).

System set-up is indicated in Figure 6.1. The six-port switching valve should be set to give two situations:

1. Flow from pump 1 is: extraction column → valve → CE column → UV detector → waste. Flow from pump 2 is: valve → analytical column → EC detector → waste.
2. Flow from pump 1 is: extraction column → valve → UV detector → waste. Flow from pump 2 is: valve → CE column → analytical column → EC detector.

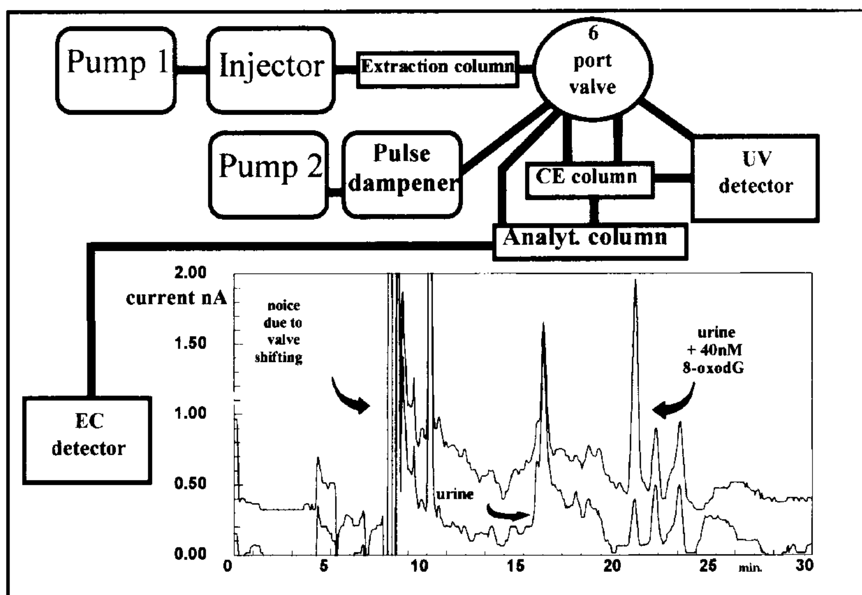

Figure 6.1 HPLC–EC analysis system set-up

- The brand of the HPLC hardware components can be chosen ad lib. However, in our experience the electrochemical detector brand suggested is the most stable and reliable among the ones tested and it gave the most reproducible results.
- When setting up a new cell for electrochemical detection, record a dynamic voltamogram (160–400 mV) by injecting an 8-oxodG sample repeatedly and changing the voltage on electrode 2 in increasing steps for recording the response on the detector. The voltamogram is an S-curve giving the relationship between applied voltage and response; setting of working potential should correspond to the first part of the upper part of the S-curve. This procedure should be repeated at regular intervals. Deterioration of the cell is seen as a right displacement of the voltamogram.
- The 5011 cell has two voltages, to be set corresponding to a first and second set of electrodes. Set the first voltage at about

100–120 mV and record the signal from the second electrode according to the voltamogram (this will reduce noise from substances that could give background interference from being electrochemically active at low voltage).

- **Flushing of the cell with 200 μM ascorbic acid can revive the cell to some extent, but replacement is needed when the voltamogram does not show a plateau or shifts to the right.**

The HPLC system is started with the six-port switching valve set for situation (1). Urine spiked with 2–4000 nM 8oxodG solution is injected for determination of the retention time until elution from the extraction and CE columns on to the UV detector. The high concentration is used to give a detectable signal on the UV detector. An electrochemical detector can be used instead of the UV detector but is not as cost-effective.

When the retention time of 8oxodG from the CE column is determined, the system is set to run in situation (1) until 1 min prior to elution; it is then switched to situation (2) for 1 min, after which it is reset to situation (1) for the remainder of the run. This will ensure that only the peak of interest (i.e. the 8oxodG peak) is diverted to the analytical column. The shift in flow/pressure during the valve shifting gives rise to large signals on the EC detector; however, this does not disturb the quantification of the 8-oxodG peak. The urine samples may give some variation in the retention time of the CE column and must be checked and set individually.

Quantification is by spiking the urine sample to be analysed with two different concentrations of pure 8oxodG.

Calculation

The 8-oxodG peak height P1 are recorded after injection of pure urine; P2 and P3 are recorded after urine has been spiked with 8-oxodG. .

The concentration in the pure urine sample is calculated twice, from each of the spiked samples:

$$C1 = P1 \times (P2 - P1)/20$$

$$C2 = P1 \times (P3 - T1)/200$$

The average, (C1 + C2)/2, is used as the measured concentration; however, C1 and C2 should not differ by more than 10%. A greater difference indicates

that separation on the analytical column is not satisfactory and that the mobile phase needs adjustments. Only trial and error can indicate how to adjust the acetonitrile concentration (most often somewhere between 2.5 and 4%). Minor adjustment of pH can also bring about satisfactory separation.

Reagents
- 8oxodG standard (Sigma SKJ 7700, molecular weight 283.2 g/mol)
- Tris (Sigma T-1503 Lot 55H55703)
- Boric acid (Riedel-de Haën, Holland 31146)
- Ortho-phosphoric acid (Riedel-de Haën, Holland 30417)
- Millipore filters (0.45 μm, cat HVLPO 4700 lot R8AM444479)

Mobile phase on pump 1
One litre of mobile phase is made from:

- 25 ml acetonitrile, HPLC grade
- 15 ml methanol, HPLC grade
- 960 ml double distilled water (or HPLC grade)
- 1.237 g sodium borate

Adjust pH with NaOH to 7.9, de-gas and filter through millipore filters (0.45 μm).

Mobile phase on pump 2
One litre of mobile phase is made from:

- 25–40 ml acetonitrile, HPLC grade (adjust as indicated from C1 and C2 concentrations as described above)
- 6.7 ml orthophosphoric acid
- Water, double distilled or HPLC grade: add 1 l (last 10–20 ml added after pH adjustment)

Adjust pH with 5 N NaOH to 2.1 (usually requires about 5 ml for a start).

Preparation of urine samples

Fresh urine, or urine samples stored at −20°C, can be used. No preservation is necessary for storage and 8oxodG is stable for years. The following procedure can be applied before or after storing the sample.

Reagents
- Tris solution, 1 M: 6.055 g in 100 ml double distilled water

Procedure

1. Use 2 ml of urine (fresh or stored).
2. Add 40 μl 2 M HCl to ensure acidic urine, and freeze.
3. Thaw sample and centrifuge at 3000 g for 10 min.
4. Mix 1.7 ml of supernatant with 34 μl 2 M NaOH.
5. Mix 95 μl supernatant and 5 μl 8-oxodG (Sigma, St Louis, Missouri) 0 nM in double distilled water (sample **S1**).
6. Mix 95 μL supernatant and 5 μl 8-oxodG 200 nM in double distilled water (sample **S2**).
7. Mix 95 μl supernatant and 5 μl 8-oxodG 2000 nM in double distilled water (sample **S3**).
8. Mix each of the three samples (**S1**, **S2**, **S3**) with 100 μl Tris buffer, pH 7.9, resulting in final spiking with 20 and 200 nM 8-oxodG.
9. Inject 25 μl of each of the three samples on to the HPLC system.

Alternative HPLC–EC procedures

Some researchers have suggested the use of two separate HPLC systems: the first for collection of the peak of interest, which is evaporated and then injected on to a second HPLC system with different elution composition. Variations in the switching system and set-up are also used (Tagesson *et al.*, 1995) and appear to be equally functional as the system described here.

Immunoaffinity columns have also been used (Shigenaga *et al.*, 1994) but such columns are not commercially available and require labelled standards to estimate recovery.

Quality control

The described assay should give linear response curves to injection of standard solutions of 8-oxodG. The inter-assay coefficient of variation should be less than 9–13% and the limit of detection should be about 0.2 nM based on a signal to noise ratio of 1:3.

Complete separation of the 8-oxodG peak can be ensured from the lack of difference between the two calculated concentrations C1 and C2. The procedure is given above under calculations.

Final calculation

Excretion of 24-hour urinary 8-oxodG is calculated by multiplying the estimated urinary concentration of 8-oxodG with the 24–hour urine volume,

giving a unit of nM/24 h. The excretion rate can also be measured per kilogram body weight, giving a unit of nM/24 h/kg BW.

Urine collection period can be varied; however, it has to borne in mind that the half-life of injected 8-oxodG from plasma is about 2–3 h. Because of this, reduction of collection time to less than 9–12 h cannot be recommended. Other means of 'biological average' can be used; for example, three overnight urine collections, or 48 h collection.

Correction of spot urine samples by estimation of urinary creatinine concentration cannot be recommended.

LCMS–MS ANALYSIS OF 8-OXODG IN URINE

Introduction

Electrochemical detection is renowned for very high sensitivity compared with UV detection and also sensitivity better or equal to that achievable by mass spectroscopy. HPLC–EC is certainly the most widely applied method for measuring oxidative damage to DNA, but, the methodology also has some limitations. The HPLC–EC method described above requires re-run of the sample and runs of additional spiked samples. Separation of 8-oxodG from other compounds in urine is quite difficult and the complicated set-up with column switching, although automated, requires human surveillance and multiple repeated adjustments. At best the capacity is up to five samples per day, and in our experience it is fewer than 500 samples per working year, even with the dedication of a full HPLC system and a technician. Furthermore, the assay is not easily extended to measure other oxidized nucleosides or the oxidized bases.

Gas chromatography–mass spectrometry (GCMS) has been used for measuring oxidative DNA modification, but extensive work-up and derivatization of samples is necessary for this type of analysis. It has been argued and also demonstrated that certain of these procedural steps can induce oxidation of non-oxidized DNA bases/nucleosides, giving rise to false high values—particularly in DNA extracts.

Coupling liquid chromatography (LC) with mass spectroscopy is a way to avoid these problems. However, the peaks resulting from LC do not match the height and narrowness of those from GC, and the single quadruple is not sensitive and selective enough to match the sensitivity of HPLC–EC.

To overcome these limitations we have started to develop an LCMS–MS method for analysis of urinary 8-oxodG, with a potential for simultaneous

measurement of other excreted DNA repair products (e.g. 8-oxoGua, 8-oxoAdenine and 8-oxodA) and with a potential for measuring DNA samples from extracted tissue.

Two different types of tandem mass spectrometers are available as bench-top machines: triple quadruples and ion trap, the latter with the potential for MS^N mass spectroscopy. Each type provides advantages and disadvantages. Ion trap mass spectrometers are particularly renowned for high sensitivity in full scan mode and are therefore particularly suited for identification purpose. In practice it is difficult to exploit the theoretical MS^N capability for N larger than 2. When it comes to quantification and analysis in SRM (selected reaction monitoring) mode, the triple quadruples are better suited and are renowned for high sensitivity in SRM mode.

For development of the LC part of the urinary assay for 8-oxodG, several columns were tested for qualities such as separation of oxidized and non-oxidized base and nucleoside and relatively short retention time, with a mobile phase without involatile buffers. The best suitable column in our experience is as indicated below.

Instrumentation set-up

Liquid chromatography

- Hewlett-Packard LC model 1050 series (HP1050)

Tandem mass spectrometer

- Perkin-Elmer SCIEX API 365 LC/MS/MS (API365) with Turbo, Ionspray®, and Perkin-Elmer software running on an Apple Power Macintosh 7300/200.

HPLC packing pump

- Magnus Scientific P6060 HPLC Slurry Packer, connected to a 200 ml stainless steel chamber for vertical packing.

A 50 × 2.0 mm i.d. steel column was packed with Waters Spherisorb S3 C6 material from a Waters Spherisorb 4.6 mm i.d. 15 cm column (part no. PSS839999, Batch 1003). Packing can be done either with methanol or (probably better) with acetone.

Preparation of 1 l of mobile phase for the LC

- 20 ml HPLC grade acetonitrile
- 1 ml acetic acid

- Add double distilled water (1 l) and adjust pH to 4.0 with ammonia solution (148 ml ammonia to 1000 ml double distilled water).

Interface between HP1050 and API365

The HP1050 is connected to the S3C6 column eluted at a flow rate of 0.2 ml/min. Methanol (flow 0.1 ml/min) is added to the effluent (flow 0.2 ml/min) before it enters the mass spectrometer (by means of a Merck Hitatchi L-6000 pump). Addition of methanol improves the sensitivity, presumably by increasing ionization.

Experience

The LCMS–MS method is not fully developed at present but sufficiently documented for the suggested set-up to be operational. The scan in Figure 6.2 of a 1000 nM sample (six scans) shows that the dominant product ion from the precursor ion at m/z 284 is m/z 168 from the precursor at m/z 284. This corresponds to selection of the protonated 8-oxodG nucleoside in Q1, breaking of the base–sugar bond NQ2 and selection of the resulting protonated base in Q3.

Figure 6.3 gives mass chromatographs of a normal human urine sample (same urine injected twice, no preparation) and the run of a 1000 nM aqueous

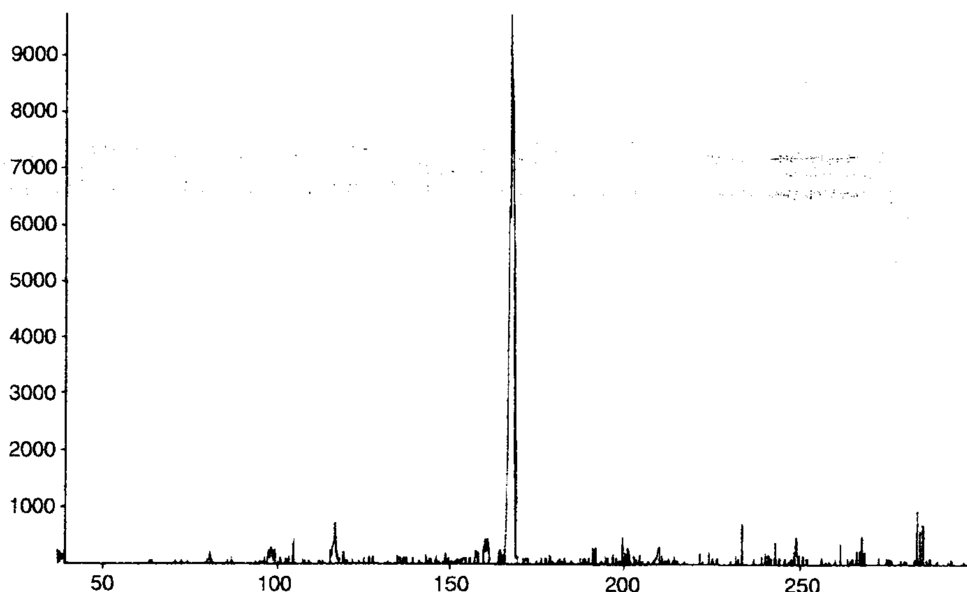

Figure 6.2 LCMS–MS results

8-oxodG sample (dashed line). As indicated by the retention time and the peak shape, there is identity of the genuine sample and the urine measurement. From time 0 to 2.2 min during which 8oxoGua is detected, m/z 168 (the protonated base) is selected in Q1; and m/z 140 (the most intense product ion, scan not given) is selected in Q3 for the remaining time that 8-oxodG is detected.

At present, internal standardization is not implemented and isotope-labelled 8-oxodG is not commercially available. The urine sample in Figure 6.3 corresponds to a concentration of about 40 nM, as judged from injection of aqueous 8-oxodG in concentrations of 1–1000 nM (correlation coefficient of this standard curve is 0.9999). Preliminary investigations on spiked urine samples show a sensitivity loss in urine of about 2.5, which clearly demonstrates the need for internal standardization.

Interpretation of urinary measurements

Estimation of urinary 8-oxodG excretion provides an estimate of whole-body oxidative stress to DNA, and as such is a rate measurement. It is

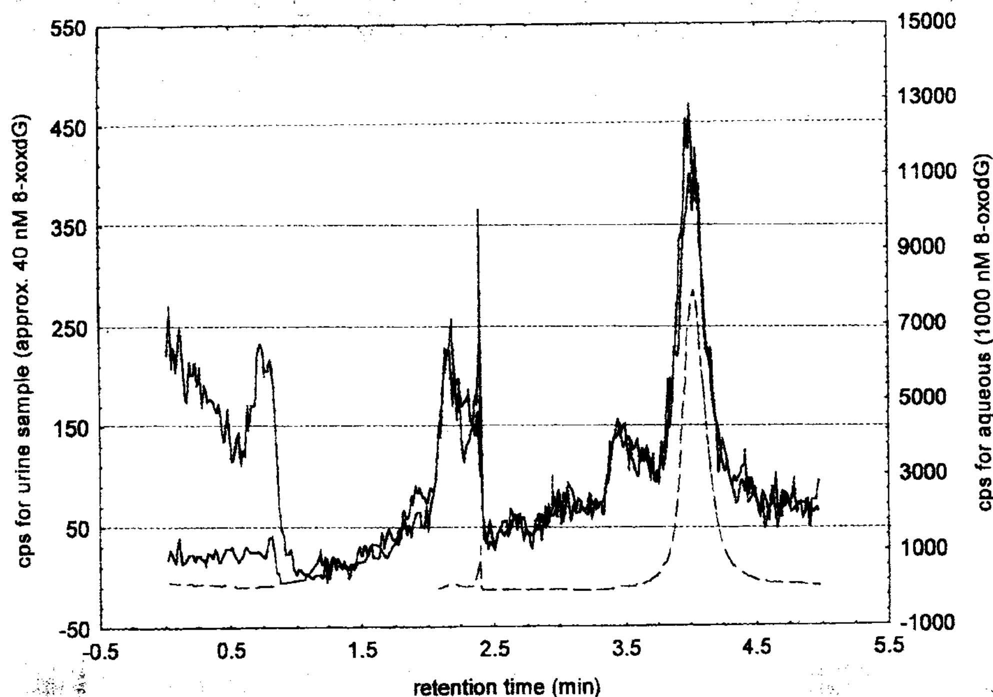

Figure 6.3 LC–MSMS of urinary 8oxodG

independent of DNA repair. This should not be confused with the concentration measurement of DNA oxidation in tissue or cell samples. Choice of measurements must rely on the specific experiment and the hypothesis under investigation. More detailed argumentation is given in Poulsen *et al.* (1998).

Conclusion

LCMS–MS analysis of urine for 8-oxodG is clearly feasible and offers several advantages over the HPLC–EC method: shorter analysis time, high specificity, quantification by internal standardization, and no need for column switching. As such, it offers a high capacity analysis for use in molecular epidemiology. The disadvantage compared with the HPLC–EC method is the four-fold higher price and the need for experience in both LC and tandem mass spectroscopy.

Although not yet demonstrated the method has the potential for measuring other oxidised nucleosides and bases, and also for use in the analysis of DNA samples extracted from tissue.

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